# Report for 2005MN102B: Estrogens and Estrogenic Activity in Swine Manure

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Report Follows

**Estrogens and Estrogenic Activity in Swine Manure**

**Prinicpal Investigators**
Kuldip Kumar, Department of Soil, Water, & Climate, Univ. of Minnesota
Ashok K. Singh, Department of Veterinary Medicine, Univ. of Minnesota
Satish C. Gupta, Department of Soil, Water, & Climate, Univ. of Minnesota

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## Executive Summary

Naturally occurring estrogens in animal waste can cause negative environmental impact through disruption of endocrine system in wild life, domesticated animal, and humans. Very little information is available on the type and the extent of estrogenic activities in swine manure. This is partially due to lack of analytical ability for estrogen analysis in manures. The goal of this study was to develop procedures for analyzing different estrogens and estrogenic activities in different types of swine wastes. The wastes included samples of urine and feces from pregnant female pig, non-pregnant pig of similar age, and a boar. The ELISA and HPLC-UV based methods were developed to quantify estrone (E1), 17β estradiol (E2), and estriol (E3) and their conjugates. HPLC analysis showed many organic compounds in manure which had properties similar to that of conjugates of the parent estrogen compounds. Therefore, we concentrated on analyzing only the parent compounds E1, E2, and E3 in this study. In general, ELISA gave higher concentrations of these compounds compared to HPLC-UV analysis. The total concentration of estrogens was more in feces than in urine and followed the trend: pregnant female > non-pregnant female > boar. The concentration of various estrogens in swine waste was variable with concentrations as low as traces to 84 μg/L of E1 in manure from nursery pigs and 1398 μg/L of E2 in pits from finishing pigs. The concentrations of E2 in manure increased by as much as 50-100% on treatment with glucuronidase and sulfatases enzymes indicating that significant concentrations of conjugates were present in manure. Although these conjugates do not have much estrogenic activity, they can convert to free estrogens in manure lagoons thus leading to higher estrogenic activities.

## Introduction and Objectives:

Estrogen hormones are emerging contaminant that are attracting public attention because at low concentrations these pollutants can adversely affect the reproductive biology of vertebrates such as fish, turtle, frogs; wild animals; and humans. One source of these contaminants in water bodies is through runoff from manure-applied fields. Currently, there is limited understanding of the toxicological significance (in particular low level long-term exposure) of these contaminants not only on human health but also on aquatic life. Several researchers have shown that estrogen concentrations as low as 10 – 100 ng/L in waterways can adversely disrupt the normal functioning of endocrine system of many species thus affecting their reproductive biology (Irwin et al., 2001; Hanselman et al., 2004). Swine farming is one of the major food-animal industries in the nation. In 2003, pig growers in the US had an inventory of 59.6 million pigs and hogs. Minnesota is #3 in swine production.

With wide spread scare about bird flu and consistent demand for while meat, a major increase in swine production facilities is expected over next several years. This means increased problems associated with disposal of swine waste and the risk of soil and water contamination.

Although estrogens excreted by the animals have been recognized as a potential pollutant, research data is lacking to make scientific assessment of the scope of estrogen contamination. An important reason for this lack of scientific data is an absence of analytical methods for the screening and conformation of estrogens, especially the conjugated estrogens, in swine waste. On an average, a pig produces about 3.9 kg of manure (a slurry of urine and feces)/day (Hamilton et al., 2003) which mean about $2.3 \times 10^8$ kg of manure is generated each day in the US by the pig industry. Furthermore, studies have shown that potent estrogens such as estrone (E1), estradiol (E2), and estriol (E3) are excreted into the environment in the urine and feces of all farm animals (Hanselman et al., 2003).

Estrogens are excreted in two forms: free estrogens such as E1 and E2 that are mostly present in feces, and conjugated estrogens such as E1S, E2S, E1G, and E2G that are mostly present in urine. Thus, stored manure generally contains both forms of estrogens. Unlike for free estrogens, the fate of conjugated estrogens in waste is not fully understood. Waste microorganisms, via their glucuronidase enzyme, have been shown to hydrolyze estrogen-glucuronide conjugate into free estrogens (Dray et al., 1972). It has also been shown that free-estrogens, and not their conjugated forms, are bioactive at very low concentrations (1 to 100 ng/L) (Matthiessen and Sumpter, 1998). Also, the potency of E2 is several folds greater than E1 and E3.

The goal of this study was to develop new methods and/or validate existing methods for detection and confirmation of estrogens, and then use the validated methods to evaluate the levels and fate of estrogens in swine manure. Specifically, the objectives were:
1. Develop ELISA methods for screening and HPLC-UV method for confirmation of free and conjugated estrogens in swine manure.
2. Develop methods for hydrolysis of conjugated estrogens using enzymes.
3. Develop methods to quantify estrogenic potency of manure samples using estrogen-receptor positive cells.

**Materials and Methods:**

**Objective 1:** Following ELISA kits were obtained from Japan Envirochemicals, Ltd, Japan to analyze estrogens in urine and manure of swine:

***Total Estrogen ELISA*:** This kit measured total (estrone E1, estradiol E2 and estriol E3) estrogens in aqueous samples. The analysis is based on a competitive reaction where enzyme labeled standard competes with free estrogens in the sample for binding to a specific monoclonal antibody immobilized to the surface of the microtiter plate. The amount of labeled estrogen bound to the plate is determined by addition of a non-colored substrate, which is converted into a colored product. The color intensity is measured at 450 nm and is inversely proportional to the amount of estrogen in the sample. The assay is calibrated using a standard solution of estrogens supplied with the kit. The assay is highly sensitive, simple and rapid to perform. The standard curve working range is 0.1-3.0 μg/L. Before ELISA test, a simple solid phase extraction was performed on urine, feces or manure samples as described in the kit. Three kits used in estrogen analysis were: (i) total estrogens (E1+E2+E3), (ii) E1

kit, and (iii) E2 kit. The amount of E3 was then estimated by subtracting the concentration of E1 and E2 from total estrogens concentrations. The kit exhibited excellent linearity and recovery for all three estrogens in free or conjugated forms.

***Individual estrogen ELISA*:** The positive samples were analyzed by using the estrogen specific kits for further identification. These kits did not distinguish between free and conjugated estrogens. When added to manure samples, different estrogens exhibited variable recoveries (25 to 50%), possibly due to their binding to the manure's solid particles.

*HPLC*-UV: Since the ELISA method did not differentiate between free and conjugated estrogens, this was achieved through the use of HPLC-UV technique. We are able to detect both free and conjugated estrogens simultaneously. Since the HPLC-UV is less sensitive than the ELISA methods, 5 to 10 ml of each sample was pooled and extracted with solid-phase extraction prior to estrogen analysis.

**Objective 2:** It is well established that the free, and not the conjugated estrogens are biologically active. Furthermore, the soil enzymes can convert conjugated estrogens into free estrogens, thus increasing the samples estrogenicity. Therefore, to determine the total estrogenic potential of a sample, it is important to convert the conjugated estrogens into free estrogens prior to analysis. To achieve the second objective, we spiked the manure samples with glucuronide and sulfate (5 ng each) conjugates of each estrogen and then incubated the samples in a mixture containing glucuronidase and sulfatase enzymes at 37 °C for 24 h. As shown in Figure 1, the conjugated estrogens were effectively converted into free estrogens thus facilitating the quantification of total estrogenic potential of four types of swine manures samples.

Briefly, the procedure for hydrolysis of conjugated estrogens into free-estrogens in swine manure involved drying the extract containing conjugated estrogens at 55 °C under nitrogen, dissolving the dried extract in 5 mL 0.2 M sodium-acetate buffer, and then incubating it with 100 µl β-glucuronidase and sulfatase enzymes (H2 type, activity 100,000 E/mL; sulfatase acivity 5000 E/mL, respectively) at 37 °C for 24 h. The hydrolyzed extract is then extracted with a combination of C18 and $NH_2$ columns and analyzed either with ELISA or HPLC-UV. The specificity of the method is determined by incubating the samples with the ß-glucuronidase inhibitor *d*-saccharic-1-4 lactone (100 mmol/L) prior to adding glucuronidase. The procedure described above yields the following fractions from each sample: (1) Fraction containing free estrogens (E1, E2, E3, 16-OH-E1, and 6-OH-E1). (2) Fraction containing conjugated estrogens (E1G, E2G, E1S, E2S, and E3G). (3) Hydrolyzed fraction containing free estrogens.

**Objective 3 (in progress):** We could not calibrate this method in time to perform this assay on manure samples in this project. However, we are in the process of growing an estrogen-positive cell lines for the purpose of determining the samples estrogenic potency. We expect to complete the developmental work for this project over next two months.

**Results:**

A comparison of ELISA and HPLC-UV results shows that solvent extraction of feces, urine, and manure slurries was sufficient to quantify the free estrogens in manure samples (Tables 1 and 2). Many organic compounds present in the manure eluted early during HPLC

determination and thus interfered with the separation of conjugated estrogens peaks. Thus, further testing of the methods was done only for free estrogens in manure and urine samples. We are continuing with efforts to develop clean up procedures for separating these conjugate estrogens peaks for swine manure samples. However, for this report only the data for free estrogens E1, E2, and E3 are reported.

In general ELISA and HPLC-UV techniques gave similar trends with ELISA giving higher concentrations of estrogens in fresh feces, urine, and manure samples. The reason for these differences may be that ELISA is based on immunological and colorimetric reaction test and conjugated estrogens and/or degradation products may be reacting with antibodies thus giving higher concentrations. Both ELISA and HPLC-UV techniques showed that there was a large variation in the concentrations of a given estrogen between the pigs urine and feces obtained from different facilities (Tables 1). For example, the concentrations of E2 in feces ranged from 1498 to 2022 μg/L as compared to 180 to 490 μg/L in urine samples using the ELISA test (Table 1). In general, higher concentrations of free estrogens were present in feces than urine and followed the trend: pregnant pigs > non-pregnant pigs > boar (Table 1).

The estrogen concentrations in actual manure samples also varied widely, with only trace concentrations of E2 in manure samples from nursery pigs to as high as 1398 μg/L in manure samples obtained from pits of a feeder-finish facility (Table 2). In general, solid manure samples obtained from hoop structures contained lower concentrations of estrogens compared to the manure obtained from lagoons or pits. This may be due to three reasons (i) better composting of solid manure leading to rapid decay of these compounds, (ii) estrogens from urine percolated below the bedding materials into the soil, and (iii) low recoveries of estrogens from solid manures (mixture of bedding and feces) as compared to liquid manures from pits and lagoons.

We also tested the hypothesis that conjugated estrogens (no significant estrogenic activity) may be present in manures. This was done through the addition of β-glucuronidase and sulfatase enzymes. The results showed that concentration of estrogen E2 increased from as much as 40-100% in the four manures when treated with β-glucuronidase and sulfatase (Figure 2). This shows that in addition to free estrogens, concentrations of conjugated estrogens may be significant in manures and needs further quantification. During manure storage in pits or lagoons, there is a potential that some of the conjugated estrogens may be converted back to free estrogens and thus increasing the estrogenic activity of manure.

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2. Publications:

Kumar, K., Satish Gupta, Ashok Singh, Shveta Gupta, Yogesh Chander, Lakhwinder Hundal, Albert Cox, and Thomas Granato. 2006. Occurance of Estrogenic Compounds in Manures and Biosolids. Invited Paper in Special Symposium on Emerging Contaminants and Land-applied Biosolids and Manures: State of the Science and regulatory implications. ASA-CSSA-SSSA International Annual Meetings. November 12-16, 2006.

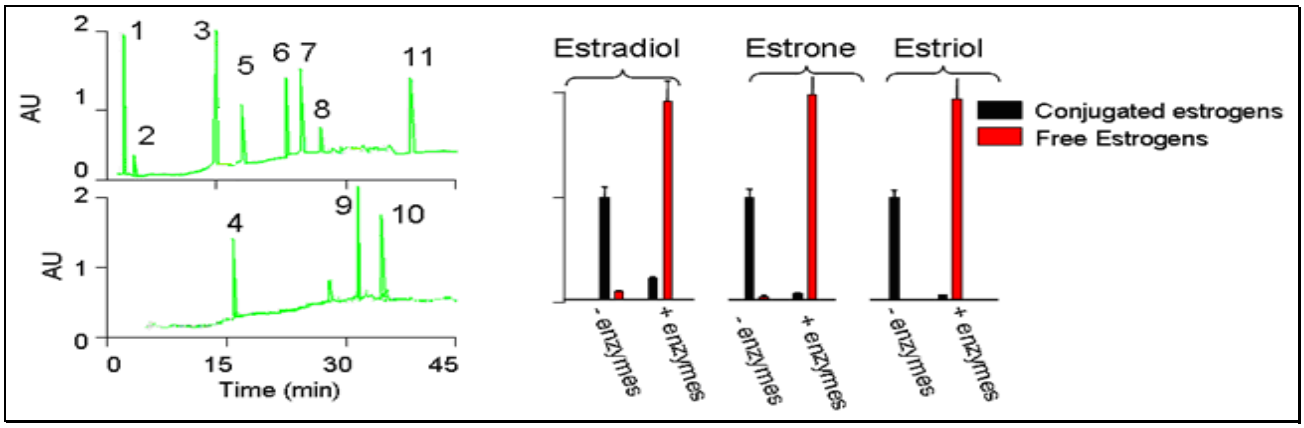


***Figure-1****: Hydrolysis of conjugated estrogens using a mixture consisting of glucuronidase and sulfatases. Top chromatograph shows conjugated estrogens and bottom chromatograph shows the enzyme hydrolyzed free estrogens. Almost 100% of the conjugated estrogens were hydrolyzed by the present method. 1 E3G, 2 E3S, 3 E2G, 4 E3, 5 E1G, 6 E2S, 7 E1S, 8 unknown, 9 E2, 10 E1, 11: unknown.*

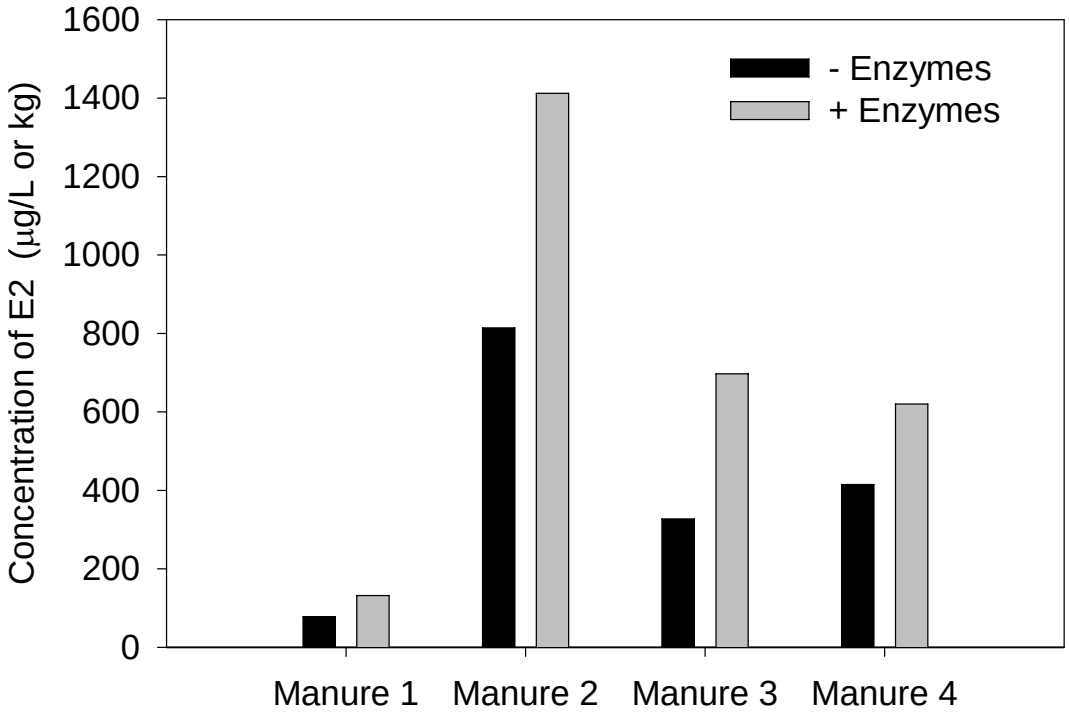


***Figure 2:*** *Hydrolysis of conjugated estrogens present in four swine manure samples using a mixture consisting of glucuronidase and sulfatase.*

**Table 1: Concentration of E1, E2, and E3 in feces and urine obtained from pregnant female pig, non-pregnant female pig and a boar using ELISA and HPLC-UV analysis.**

| | Feces (μg/kg dry weight) | | | | | | Urine (μg/L) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Pregnant Pig | | Non-Pregnant | | Boar | | Pregnant Pig | | Non-Pregnant | | Boar | |
| | ELISA | HPLC | ELISA | HPLC | ELISA | HPLC | ELISA | HPLC | ELISA | HPLC | ELISA | HPLC |
| E1 | 2409 | 2350 | 2221 | 2418 | 1127 | 1188 | 780 | 598 | 430 | 348 | 424 | 455 |
| E2 | 2022 | 1790 | 1871 | 1316 | 1498 | 830 | 490 | 792 | 378 | 180 | 180 | 66 |
| E3 | 3478 | 3380 | 1078 | 998 | 974 | 524 | 1084 | 898 | 904 | 504 | 90 | 26 |
| Total | 7909 | 7520 | 5170 | 4732 | 3599 | 2542 | 2354 | 2288 | 1712 | 1032 | 694 | 547 |

**Table 2: Concentration of various forms of estrogens in swine manures obtained from different facilities.**

| Estrogen | Nursery pigs (n=2) | | Finishing hoops (μg/kg) (n=3) | | Feeder-Finish pits (μg/L) (n=3) | | Finishing lagoons (μg/L) (n=3) | |
|---|---|---|---|---|---|---|---|---|
| | ELISA | HPLC | ELISA | HPLC | ELISA | HPLC | ELISA | HPLC |
| E1 | 84±37 | ND | 149±56 | 90±45 | 648±319 | 455±203 | 1098±312 | - |
| E2 | Traces | ND | 85±42 | 42±32 | 1398±418 | 915±157 | 327±156 | - |
| E3 | Traces | ND | 203±72 | 104±56 | 58±26 | 43±18 | 208±92 | - |

**ND – Not detected; - Not analyzed.**